Short Communication

ATIC-ALK: A Novel Variant ALK Gene Fusion in Anaplastic Large Cell Lymphoma Resulting from the Recurrent Cryptic Chromosomal Inversion, inv(2)(p23q35)

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The subset of CD30-positive anaplastic large cell lymphomas (ALCL) with the *NPM-ALK* gene fusion arising from the t(2;5)(p23;q35) forms a distinct clinical and prognostic entity. Recently, various cytogenetic, molecular, and protein studies have provided evidence for the existence of several types of variant *ALK* fusions in up to 20% of *ALK*+ ALCL, of which only one, a *TPM3-ALK* fusion resulting from a t(1;2)(q25;p23), has so far been cloned. A cryptic inv(2)(p23q35) has been described as another recurrent cytogenetic alteration involving *ALK* and an unidentified fusion partner in some ALCL. In a screen for variant *ALK* gene fusions, we identified two ALCL that were negative for *NPM-ALK* by reverse transcriptase-polymerase chain reaction, but were positive for cytoplasmic *ALK* with both polyclonal and monoclonal antibodies to the *ALK* tyrosine kinase domain, consistent with *ALK* deregulation by an alteration other than the t(2;5). Case 1 was a T-lineage nodal and cutaneous ALCL in a 52-year-old woman, and Case 2 was a T-lineage nodal ALCL in a 12-year-old girl. FISH analysis confirmed *ALK* rearrangement in both cases. An inverse polymerase chain reaction approach was then used to identify the *ALK* translocation partner in Case 1. We found an in-frame fusion of *ALK* to *ATIC*, a gene previously mapped to 2q34-q35. We then confirmed by DNA polymerase chain reaction the localization of *ATIC* to yeast artificial chromosome (YAC) 914E7 previously reported to span the 2q35 break in the inv(2)(p23q35). FISH analysis in Case 1 confirmed rearrangement of YAC 914E7 and fusion to *ALK*. The

***ATIC-ALK* fusion was confirmed in Case 1 and also identified in Case 2 by conventional reverse transcriptase-polymerase chain reaction using *ATIC* forward and *ALK* reverse primers. *ATIC* encodes an enzyme involved in purine biosynthesis which, like other fusion partners of *ALK*, is constitutively expressed and appears to contain a dimerization domain. *ATIC-ALK* fusion resulting from the inv(2)(p23q35) thus provides a third mechanism of *ALK* activation in *ALK*+ ALCL. (Am J Pathol 2000, 156:781-789)**

Anaplastic large cell lymphoma (ALCL) is recognized as a distinct subtype of non-Hodgkin's lymphoma (NHL) in recent lymphoma classifications. This disease constitutes approximately 5% of all NHL but accounts for 30 to 40% of pediatric large cell lymphomas.¹ ALCL expresses Ki-1 (CD30), an antigen originally detected on Reed-Sternberg cells of Hodgkin's disease, later shown to be a member of tumor necrosis factor receptor family.^{2,3} In its classical or common form, ALCL shows an often bizarre, anaplastic morphology with sinusoidal infiltration of lymph nodes and a pseudocohesive appearance, and T or null phenotype.⁴ The status of ALCL as a discrete entity had long been controversial,^{5,6} and its recent separation into at least two subsets (see Discussion) stems from cytogenetic and molecular studies of the translocation seen in about 40 to 60% of cases, t(2;5)(p23;q35).⁷⁻⁹

In 1994, the t(2;5) was found to involve a novel gene at 2p23 encoding a tyrosine kinase, *ALK* (anaplastic lymphoma kinase), and the *NPM* (nucleophosmin) gene at

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5q35, which encodes a nucleolar phosphoprotein.¹⁰ The resulting fusion gene encodes a chimeric protein, NPM-ALK, with a molecular weight of 80 kd, consisting of the N-terminal portion of NPM fused to the catalytic domain of ALK.¹⁰ ALK is a tyrosine kinase receptor belonging to the insulin growth factor receptor superfamily, highly related to the leukocyte tyrosine kinase (LTK) gene but normally expressed only in the nervous system.^{11,12} The fusion with *NPM* contributes the *NPM* promoter and the NPM oligomerization domain to NPM-ALK, and removes the ALK extracellular and transmembrane domains. As a result, the ALK kinase domain within NPM-ALK is constitutively activated through autophosphorylation, and its expression is deregulated and ectopic, both in terms of cell type and cellular compartment (cytoplasm; reviewed in Ref. 13). Downstream targets of the ALK kinase domain that may be relevant in mediating the oncogenicity of NPM-ALK are being identified.¹⁴

Because of the highly restricted expression of native *ALK* in the nervous system and its absence in normal lymphoid tissues, immunohistochemical detection of aberrantly expressed ALK protein using monoclonal^{15,16} or polyclonal^{17,18} antibodies to the ALK kinase domain (retained in NPM-ALK) was found to be a sensitive and specific method for detecting *NPM-ALK*-positive ALCL. Interestingly, ALK immunostaining was observed in both cytoplasm and nucleus in most cases, but only in cytoplasm in some cases. The nuclear localization of NPM-ALK is due to the formation of heteromeric complexes with native NPM, which contains a nuclear localization signal.¹⁴ Initially, the occasional variability in subcellular localization of ALK immunostaining was thought to reflect unknown factors affecting either the heteromerization of NPM-ALK with NPM, or the entry of the resulting heteromeric complexes into the nucleus. However, it soon became apparent that ALCL with exclusively cytoplasmic ALK immunoreactivity usually lacked *NPM-ALK* by reverse transcriptase-polymerase chain reaction (RT-PCR).^{15,16,19} At the same time, using an artificial *TPR-ALK* construct, it was shown that only cytoplasmic localization is required for transformation by the ALK portion of NPM-ALK.²⁰ Taken together, these results suggested that in some ALCL, *ALK* may become oncogenically activated through fusion with other translocation partners unassociated with nuclear transport.

Studies of large series of Ki-1 ALCL by ALK immunostaining now indicate that up to 20% of cases show cytoplasmic staining only.^{16,21,22} Furthermore, Western blot analysis has identified at least four different types of aberrant *ALK*-positive proteins in different cases of this subset of Ki-1 ALCL that show ALK immunostaining confined to the cytoplasm.²³ Thus, up to 20% of ALK+ Ki-1 ALCL may contain variant *ALK* translocations, and these may be of at least four types. By cytogenetic analysis, several variant translocations involving 2p23 have been reported in Ki-1 ALCL. These include t(2;13)(p23;q34),²⁴ t(1;2)(q25;p23),²⁵ a cryptic inv(2)(p23q35),²⁶ t(1;2)(q21;p23), and t(2;3)p23;q21.²⁷ Of these, only the t(1;2)(q25;p23) has so far been cloned. Using a PCR-based genomic walking technique, Lamant et al²⁸ demonstrated that the gene involved at 1q25 is *TPM3*, which

Table 1. Primers

Primer	Sequence
NPM-5'	TCCCTTGGGGGCTTTGAAATAACACC
ALK-3'	CGAGGTGCGGAGCTTGCTCAGC
ALKREV	GCAGTAGTTGGGGTTGTAGTC
ALKREV3	TGCAGCTCCTGGTGCTTCC
ALKREV4	TGGTGCTCCGGCGGTAC
ALKFWD4	ACAAGCTGAGCAAGCTCCGC
ALKFWD5	ACCTCGACCATCATGACC
ATIC-FWD	CACGCTCGAGTGACAGTG
ATIC-REV	TCGGAGCTCTGCATCTCCG
PGK-FWD	CAGTTTGGAGCTCCTGGAAG
PGK-REV	TGCAAATCCAGGGTGCAGTG

All sequences are 5' to 3'.

encodes nonmuscular tropomyosin and was previously known to similarly rearrange with and activate the NTRK1 receptor tyrosine kinase in some papillary thyroid carcinomas (reviewed in Ref. 29).

In the present report, we describe the cloning of a novel variant *ALK* gene fusion, *ATIC-ALK*, which is associated with the previously reported recurrent cryptic inversion, inv(2)(p23q35).

Materials and Methods

Patient Samples

Among 26 cases of ALCL diagnosed at Memorial Sloan-Kettering Cancer Center that had material available for molecular studies, we identified 13 cases (50%) negative for *NPM-ALK* by reverse transcriptase-polymerase chain reaction (RT-PCR), performed as reported previously,⁹ using the primers *NPM-5'* and *ALK-3'* listed in Table 1. Molecular data on 10 of 13 *NPM-ALK*+ and 8 of 13 *NPM-ALK*- cases have been described in part in previous studies.^{9,30,31} All cases were immunohistochemically positive for Ki-1 antigen (CD30) using monoclonal antibody Ber-H2 (Dako, Santa Barbara, CA). Lineage phenotype and genotype were determined according to standard immunophenotypic and molecular genetic methods, as described in more detail elsewhere.⁴ Case histories of the two patients that were studied in more detail (see Results) are summarized below.

Case Summaries

Case 1

This 52-year-old woman was diagnosed overseas with malignant lymphoma in a left axillary mass and was treated with four cycles of chemotherapy without response. She came to MSKCC 4 months later for a second opinion. Clinical restaging showed left axillary adenopathy, focal infiltration of fat and adjacent muscle, and retraction and thickening of overlying skin. Bone marrow biopsy did not show infiltration by lymphoma. Biopsies of axillary and skin tumors showed large, polymorphic cells, with amphophilic cytoplasm, round and lobulated nucleus with two or three nucleoli. Frequent mitotic figures,

Figure 1. Representative histological appearance and ALK immunohistochemistry in Cases 1 and 2. Both cases showed strong cytoplasmic, but not nuclear, immunoreactivity with the ALK-11 polyclonal antibody and the ALK-1 monoclonal antibody, both generated to the same peptide from the C terminal portion of ALK retained in ALK fusion proteins.

necrosis, and phagocytosis were also seen (Figure 1). The tumor showed the following staining features: CD30+, epithelial membrane antigen (EMA) +, CD43+, CD3-, CD45RO-, CD20-. No clonal rearrangement involving the immunoglobulin heavy chain gene (*IGH*) was detected by Southern blotting, but the *TCR β* gene did show clonal rearrangement. This pattern was consistent with a Ki-1-positive T cell ALCL. Cytogenetic analysis of this biopsy showed the following clonal karyotype: 46, XX, del(1)(p32), der(3)dic(1;3)(q13;q27), der(6)t(1;7;6)(q25;?; p25), hsr(7)(p22), l(8)(q10), der(12)t(9;12)(q13;p13), add(13)(p11), der(15;15)(q10;q10), add(16)(q22), add(19)(q13), add(21)(p11), +mar. The patient returned overseas and was lost to follow-up.

Case 2

At the age of 12, this girl was first diagnosed with a nodal diffuse large cell lymphoma. The clinical stage was IIIA (negative bone marrow, no extranodal sites). She was treated with chemotherapy and external radiation, and achieved complete remission. Three years later, she developed a relapse and was treated with a similar chemoradiotherapy combination, and achieved a prolonged second complete remission. Twelve years later, she developed a second nodal relapse and started a new chemotherapy protocol. She died 3 months later due to sepsis and granulocytopenia. Biopsy of the second nodal recurrence showed rounded, monomorphic tumor cells with round nuclei and one or two nucleoli (Figure 1). Numerous mitotic figures were seen. The tumor showed the following immunostaining: CD30+, EMA+, CD45+, CD43+, CD20-, CD15-. No clonal rearrangement in-

volving *IGH* was detected by Southern blot analysis, but the *TCR β* gene was clonally rearranged. This pattern was consistent with a Ki-1-positive T cell ALCL. Cytogenetic analysis showed the following clonal abnormalities: 47, XX, +2, del(6)(q21), t(8;13;20)(p11.2;p11.2;p11.2).

ALK Immunostaining

ALCL were subjected to immunostaining with a polyclonal antibody generated to amino acid residues 419–520 of NPM ALK, designated ALK-11 (gift of S. W. Morris),¹⁸ after heat-induced epitope retrieval in citrate buffer for 10 minutes. Equivalent results were obtained at dilutions of 1:1000 and 1:2000. This antibody produced strong nuclear and cytoplasmic staining in all 5 ALCL tested that were positive for *NPM-ALK* by RT-PCR (results not shown). Cases positive with ALK-11 were further tested with the ALK-1 monoclonal antibody, generated to the same amino acid residues of NPM-ALK as the ALK-11 antibody (Dako),¹⁵ at a dilution of 1:50, after heat-induced epitope retrieval in citrate buffer for 20 minutes. Immunoperoxidase staining was performed on paraffin sections, using a standard avidin-biotin peroxidase procedure.

Fluorescence in Situ Hybridization (FISH) with ALK and 2q35 Probes

Bicolor FISH studies were performed on cytologic touch preparations of Case 1 and on extracted nuclei from paraffin-embedded tissue blocks from Case 2 and the two ALK-11+ but ALK-1- cases (see Results) using the

Vysis LSI *ALK* probe assay (Vysis, Inc., Downers Grove, IL) according to the manufacturer's instructions. In addition, FISH studies with a 2p23 (*ALK*) breakpoint spanning probe (600 kb, courtesy of Dr. J. Proffitt, Vysis, Inc.) and yeast artificial chromosome (YAC) 914E7 (a 1750-kb CEPH YAC probe mapped to 2q35, Research Genetics, Huntsville, AL) were also performed on Case 1 and FISH studies with an *ALK* P1 clone (designated *ALK*-DMPC-HFF#1-1111H1, courtesy of Dr. S. W. Morris) and 914E7 were performed on Case 2. With respect to the latter hybridizations, probe mixtures containing 200 ng biotin-labeled YAC 914E7 (Nick Translation Kit, Vysis) and Spectrum Orange-labeled 2p23 (*ALK*) breakpoint spanning probe or digoxigenin-labeled *ALK*P1 was applied to a slide and sealed under a coverslip. The cells and probes were codenatured at 85°C for 5 minutes and incubated overnight at 37°C in a humidity chamber. Detection of signals was performed as described in detail elsewhere.³² As negative controls, metaphase cells obtained from a cytogenetically normal lymph node and cytologic touch preparations of normal skeletal muscle were simultaneously hybridized with these probes.

Images were prepared using the Cytovision Image Analysis System (Applied Imaging, Santa Clara, CA). One hundred interphase nuclei with strong and well-delineated signals were examined by two different individuals. A separation of the Spectrum Orange- and Spectrum Green-labeled 2p23 breakpoint flanking probes (3' telomeric and 5' centromeric respectively to the 2p23 breakpoint of the t(2;5)(p23;q35) associated with non-Hodgkin's lymphoma; Vysis LSI *ALK* probe assay) was interpreted as a rearrangement of the *ALK* gene.

Inverse PCR

For both inverse PCR and conventional RT-PCR, total RNA was extracted using Trizol method (Tel-Test, Friendswood, TX), and the adequacy of the extracted RNA was confirmed by amplification of a 247-bp fragment of the ubiquitous phosphoglycerate kinase (PGK) transcript, using primers PGK-FWD and PGK-REV. For inverse PCR, double-stranded cDNA was synthesized as follows using a cDNA Synthesis Kit (Boehringer Mannheim, Indianapolis, IN). Reverse transcription was performed on 1 µg of RNA, and primed with 2 pMol of *ALK*REV primer using AMV reverse transcriptase. The *ALK*REV primer binds 98 bp from the *ALK* fusion point in *NPM-ALK* and *TPM3-ALK*. Second-strand cDNA synthesis was performed using *Escherichia coli* DNA polymerase I and RNase H. The resulting double-stranded cDNA was then blunt-ended with T4 DNA polymerase and subsequently purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The cDNA was then circularized by overnight incubation at room temperature in the presence of 1 U/µl T4 DNA ligase (Boehringer Mannheim) in a final volume of 30 µl. The ligation reaction was stopped by 65°C incubation for 10 minutes. The circularized cDNA was then relinearized by digestion with *Pst*I, which cuts the *ALK* cDNA between the *ALK*REV3 and *ALK*-FWD4 primer binding sites (oriented 5' end to 5' end).

After a manual hot start, the cDNA was then amplified by PCR (35 cycles: 95°C for 45 seconds, 58°C for 1 minute, 72°C for 3 minutes, final extension 72°C for 10 minutes) with primers *ALK*REV3 and *ALK*FWD4 (15 pMol of each) using 2U/µl *rTth* DNA Polymerase (Perkin Elmer, Branchburg, NJ). Nested PCR was performed on 1 µl of the first PCR product using primers *ALK*REV4 and *ALK*FWD5, *Taq* polymerase (DNA PCR Beads, Pharmacia Biotech, Piscataway, NJ), for 35 cycles (95°C for 1 minute, 62°C for 45 seconds, 72°C for 1 minute, final extension 72°C for 10 minutes).

RT-PCR for ATIC-ALK

RT-PCR was performed using ATIC-FWD and *ALK*REV primers. First, reverse transcription was performed for 30 minutes at 42°C on 1 µg of RNA using 10× buffer II, 25 mmol/L MgCl₂, 50 mmol/L random hexamers, 10 mmol/L dNTP, 40U/µl RNase inhibitor, 200U/µl reverse transcriptase, and DEPC-treated H₂O for a final volume of 20 µl. A negative control (10 µl of DEPC-water) was included at this stage. The reverse transcriptase was inactivated at 99°C for 5 minutes. Eighty µl of master mix (water, 10× buffer II, MgCl₂ for a final concentration of 1.5 mmol/L, 15 pMol of each primer, 5U/µl of AmpliTaq DNA Polymerase, Perkin Elmer, Norwalk, CT) were added to the tube. PCR consisted of 35 cycles of 95°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, final extension of 72°C for 10 minutes. The PCR products were electrophoresed in 2% NuSieve agarose gel (FMC Bioproducts, Rockland, ME) and visualized by ethidium bromide staining.

YAC DNA Extraction

YACs 914E7 and 777D5 were obtained from Research Genetics. One colony was inoculated in 5 ml YPD medium (yeast extract, peptone, dextrose; Bio 101, Vista, CA) and incubated in an orbital shaker for 24 hours at 30°C. Eight hundred µl of this product were combined with 200 µl of glycerol and stored at -70°C. The remaining product was inoculated in 100 ml YPD medium and incubated in an orbital shaker for more 24 hours at 30°C. The YAC was centrifuged for 10 minutes at 3000 rpm and the pellet was resuspended in a lysis buffer (20% sodium dodecyl sulfate, 10% Triton X-100, 5 mol/L NaCl, 1 mol/L Tris, pH 8.0, 0.5 mol/L EDTA, pH 8.0). Seventy-five microliters of glass beads and 200 µl of 1:1 phenol:chloroform were added to the lysate and it was mixed in a vortex for 5 minutes. Two hundred microliters of TE buffer (10 mmol/L Tris, 1 mmol/L EDTA) was added to the lysate and it was mixed again. After 5 minutes centrifugation at room temperature, the clear supernatant was transferred to a new tube. Then, 750 µl of 100% isopropanol was added to it, mixed gently by inversion, and left for 5 minutes at room temperature. After centrifugation, a pink pellet was seen. The dried pellet was then resuspended in 300 µl TE buffer (pH 7.4). Fifteen microliters of 1 mg/ml RNase A was added and the product was incubated for 30 minutes at 37°C. The pellet was again precipitated with 100% isopropanol and 3 Mol/L NaAc. After centrifu-

Figure 2. A: FISH studies performed on Case 1 with the LSI ALK probe assay revealed the presence of a normal chromosome 2 homologue (arrow), a rearrangement of ALK (separate orange and green signals) and an extra copy of the probe telomeric to the 2p23 breakpoint (extra orange signal). **B:** FISH studies performed on Case 1 with the 2p23 (ALK) breakpoint spanning probe and 914E7 (2q35 breakpoint spanning probe) revealed the presence of a normal chromosome 2 homologue (separate orange and green signals) and rearrangement with fusion of 2p23 and 2q35 breakpoints (juxtaposed orange and green signals). In conjunction with the findings illustrated in **A**, these results are compatible with the presence of an *inv*(2)(p23q35) with an extra copy of the ATIC-ALK fusion gene.

gation, it was washed in 70% ethanol and dissolved in TE buffer. The DNA was electrophoresed in a 1% agarose gel to evaluate its quality.

Results

Identification of Two Cases with Evidence of Variant ALK Rearrangements

The study group consisted of 13 ALCL of non-B cell lineage that lacked *NPM-ALK* by RT-PCR (see Materials and Methods). There were 7 female and 6 male patients, with median age of 47.3 years (range, 13–70 years). These 13 cases were subjected to immunostaining with polyclonal ALK-11 antibody to the ALK kinase domain. Four T cell ALCL cases were positive. These four cases were further tested by immunostaining with the ALK-1 monoclonal antibody, and by interphase FISH analysis for ALK rearrangement. Two cases, Cases 1 and 2, were also positive with ALK-1 (Figure 1). Case 1 also showed ALK rearrangement by FISH using 2p23 (ALK) breakpoint flanking probes. Specifically, a separation of these breakpoint flanking probes was detected in 97% of the interphase nuclei analyzed in Case 1 (Figure 2A) using the two-color Vysis ALK probe FISH assay, indicative of an ALK rearrangement. Moreover, a third copy of the Spectrum Orange signal of this probe set, which is located telomeric to the 2p23 breakpoint, was observed in all abnormal cells of Case 1 (Figure 2A). FISH studies with

Figure 3. Schema of inverse PCR methodology. Technical details are provided in Materials and Methods. The primers used were as follows: P1, ALKREV; P2, ALKFWD4; P3, ALKREV3; P4, ALKFWD5; P5, ALKREV4. Dimensions are not to scale, and primer positions are relative.

the two-color Vysis ALK probe FISH assay were unsuccessful in Case 2, where only extracted nuclei from paraffin-embedded tissue blocks were available. Brief case histories for these two patients are presented above in Materials and Methods. The remaining two cases that were negative by ALK-1 immunohistochemistry were also negative by ALK FISH.

Cloning of ATIC-ALK Fusion by Inverse PCR in Case 1

As schematized in Figure 3 and described in more detail in Materials and Methods, we performed inverse PCR

Figure 4. Results of inverse PCR, ATIC-ALK RT-PCR, and sequencing of RT-PCR products in Case 1. Inverse PCR performed in Case 1, as illustrated in Figure 2 and described in Materials and Methods, yielded two predominant bands. **Band A** was a nonspecific amplification product. Sequence analysis of **band B** demonstrated an in-frame ATIC-ALK fusion transcript (see Results for details). Based on this fusion transcript, the ATIC-FWD primer was designed and used in combination with the ALKREV primer in an ATIC-ALK RT-PCR assay. This yielded a 370-bp band in Case 1, but not in liver RNA. Sequencing of this band revealed an in-frame ATIC-ALK fusion transcript. The portion of the electrophoretogram containing the fusion point is illustrated. M, ϕ X174/*Hae*III size marker bands (in bp).

with nested amplification to isolate the *ALK* translocation partner in this case. There were two inverse PCR product bands: a broad 200- to 300-bp band (A), and a fainter band of approximately 120 bp (B; Figure 4). Both products were analyzed by direct automated sequencing. Sequence analysis of the 120-bp B band showed an in-frame fusion between *ATIC* and *ALK*, occurring at codon 162 of the former and codon 1058 of *ALK*, the same codon involved in the *NPM-ALK* fusion. The broad 200- to 300-bp A band was a nonspecific PCR product.

Confirmation of *ATIC-ALK* Fusion by RT-PCR in Cases 1 and 2

Based on the *ATIC-ALK* chimeric transcript identified by inverse PCR, we designed primer *ATIC-FWD* to produce a 169-bp RT-PCR product in conjunction with the *ALKREV* primer. RT-PCR with these primers yielded only a single strong 370-bp band in both cases (Figures 3 and 4), instead of the expected 169-bp product. Sequence analysis of this 370-bp band also showed an in-frame fusion between *ATIC* and *ALK*, occurring again at codon 1058 of *ALK*, but at a different point in *ATIC*, codon 229 instead of 162 (Figure 3). In light of this result, we suspect that this major fusion transcript may have been either obscured in the inverse PCR by the nonspecific 200- to 300-bp product (see above) or that the shorter fusion transcript may have been more efficiently isolated for technical reasons. This shorter fusion transcript, which was detected only in Case 1 by the nested amplification of the inverse PCR procedure, likely arose by alternative splicing of the major fusion product. The intervening portion of *ATIC* may therefore correspond to one or more exons (the exon structure of *ATIC* is largely unknown³³). This shorter minor splice form is unlikely to be biologically significant because of its low expression level and because it lacks the *ATIC* dimerization domain (see Discussion). As an incidental observation, our sequencing data confirmed that *ATIC* codon 164 reads GAC (for aspartic acid), as in reference 34, instead of GGC (for glycine) reported in reference 35. Furthermore, a search of the expressed sequence tag database (dbEST) identified five perfect matches for GAC (D83877, H09885, H06543, R20197, AA112359) and none for GGC at this codon.

To assess Case 2 for the presence of the *ATIC-ALK* fusion, we performed RT-PCR using the same primers as above, namely *ATIC-FWD* and *ALKREV*. This yielded the same 370-bp RT-PCR product (Figure 5), confirmed by

Figure 5. Detection of *ATIC-ALK* fusion transcript in Case 2. RT-PCR, using the same primers as in Case 1 (see Figure 4), demonstrated the same product in total RNA from Case 2. Controls lacking either reverse transcriptase or RNA were appropriately negative. M, 100-bp ladder size marker bands (in bp).

Figure 6. Schematic diagram of predicted *ATIC-ALK* fusion protein. The fusion protein retains the tyrosine kinase (TK) domain of *ALK* as well as the IMPCH and the linker-dimerization domains of *ATIC*. The rearrangement interrupts the AICARFT domain of *ATIC* and excludes the extracellular and transmembrane (TM) domains of *ALK*. The proportions are roughly to scale, but domain boundaries within *ATIC* are still somewhat approximate.

sequencing to be the *ATIC-ALK* fusion transcript.

Mapping of *ATIC* to a YAC Spanning the 2q35 Breakpoint in the *inv(2)(p23q35)*

ATIC was previously mapped to 2q34-q35 by FISH.³³ YAC 914E7 at 2q35 was reported by Wlodarska et al to be rearranged by the cryptic *inv(2)(p23q35)*.²⁶ To confirm that this YAC contains the *ATIC* gene, we performed DNA PCR on purified YAC DNA using primers *ATIC-FWD* and *ATIC-REV*. The expected 71-bp product was amplified from YAC 914E7 DNA, but not from an unrelated YAC (777D5 from 1q21; result not shown), confirming that *ATIC* maps to YAC 914E7.

Confirmation of *ATIC-ALK* Fusion by FISH in Cases 1 and 2

FISH studies performed on Case 1 with the Spectrum Orange-labeled 2p23 (*ALK*) breakpoint spanning probe and the biotin-labeled YAC 914E7 revealed a distinct or separate orange and green signal consistent with the presence of a normal chromosome 2 homologue and three orange and green signals lying directly adjacent or juxtaposed to each other indicative of 2p23 and 2q35 rearrangements in 96% of the interphase nuclei analyzed (Figure 2B). In Case 2, FISH analysis of nuclei from paraffin-embedded tissue blocks with *ALK* P1 and YAC 914E7 probes revealed a distinct or separate red (rhodamine) and green (fluorescein isothiocyanate) signal consistent with the presence of a normal chromosome 2 homologue and two red and green signals lying directly adjacent or juxtaposed to each other, indicative of two copies of *ATIC-ALK* fusion in 88% of the interphase nuclei analyzed (not illustrated). The limited tissue quantity available permitted analysis of 50 interphase nuclei in this case. Thus, the findings in both cases were compatible with the presence of an *inv(2)(p23q35)* and an additional copy of *ATIC-ALK*.

Discussion

ATIC (also known as hPurH) encodes 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cy-

clohydrolase (AICARFT/IMPCH), a bifunctional enzyme that catalyzes the penultimate and the final steps of the purine nucleotide synthesis pathway, AICARFT and IMPCH. As expected for an enzyme required for DNA synthesis, *ATIC* is ubiquitously expressed,³⁴ and this should provide a strong active promoter to the *ATIC-ALK* fusion gene. The promoters of the two other fusion partners of *ALK*, *NPM* and *TPM3*, are both constitutively active in lymphoid cells.²⁸ Although *ATIC* is known to be highly expressed in the CCRF-CEM leukemia cell line,³⁴ the identification of *ATIC-ALK* in ALCL may warrant a more detailed analysis of *ATIC* expression levels in lymphoid lineages.

Studies of *ATIC* deletion mutants have confirmed the existence of two non-overlapping functional domains, separated by a linker region.³³ Based on these deletion studies and on crystallography data, a working model of *ATIC* has been proposed in which residues 1 to 169 encode the IMPCH function, residues 170 to 199 encode the linker region, and residues 200 to 592 encode the AICARFT activity.^{33,35} Furthermore, crystallography and equilibrium sedimentation studies indicate that *ATIC* exists mainly as a homodimer.³³ Gel filtration and ultracentrifugation studies of additional *ATIC* deletion mutants suggest that the linker region contains a dimerization domain (G. P. Beardsley, personal communication). The first 229 amino acid residues of the predicted *ATIC-ALK* protein are identical to those of *ATIC* (the valine codon at the fusion point is conserved). Thus, in addition to an active promoter, *ATIC* appears to contribute a dimerization domain to *ATIC-ALK*, which should lead to constitutive autophosphorylation and activation of the *ALK* kinase domain. These properties are shared by *NPM* (see Introduction) and *TPM3*. In ALCL with the t(1;2)(q25;p23), *TPM3* contributes to *TPM3-ALK* an active promoter, and activation of the *ALK* catalytic domain probably results from homodimerization through the *TPM3* protein-protein interaction domain.²⁸ Like *ATIC-ALK*, *TPM3-ALK* accumulates only within the cytoplasm.^{20,28}

As discussed in the Introduction, various lines of evidence suggest that up to 20% of *ALK*+ ALCL contain variant *ALK* translocations. Furthermore, these may be of at least four types, according to the Western blot studies of Pulford et al.²³ They identified four different types of putative variant *ALK* fusion proteins with molecular weights of 85, 97, 104, and 113 kd (compared to 80 kd for *NPM-ALK* and 200 kd for native *ALK*), in three of which tyrosine kinase activity was also confirmed.²³ Whether any of these correspond to *ATIC-ALK* or *TPM3-ALK* is presently unclear. None of their cases had a documented inv(2)(p23q35), whereas one case with a aberrant *ALK* protein of 104 kd had a t(1;2), but with a 1q21 break instead of the 1q25 break associated with *TPM3-ALK*.²⁸ *NPM*, *ATIC*, and *TPM3* contribute 116, 229, and 255 amino acid residues to their respective *ALK* fusion proteins. Based on an average of 120 d per amino acid residue, the predicted molecular weight of the *ATIC-ALK* protein is about 94 kd, and that of *TPM3-ALK* is 97 kd. Aside from the approximate nature of these estimates, posttranslational modifications may alter protein mobility,

making it difficult to directly assign bands on Western blots to specific predicted fusion proteins.

The inv(2)(p23q35) was first reported in 1997¹⁹ and was then described in detail in three cases by Wlodarska et al.²⁶ This inversion is cryptic, ie, it is not apparent on conventional Giemsa-banded cytogenetic preparations, because terminal bands of similar size and staining pattern are exchanged. This may explain why no 2p23 or 2q35 breaks were apparent in the conventional karyotypes of cases 1 and 2 (see Case Summaries). Furthermore, Wlodarska et al.²⁶ found a consistent association of the inv(2)(p23q35) with a secondary chromosomal aberration, ider(2)(q10)inv(2)(p23q35), which results in extra copies of the rearranged *ALK* gene. FISH analysis demonstrated at least one additional copy of the fusion gene in both of our cases. Consistent amplification of *ATIC-ALK* suggests that it may be less oncogenic than *NPM-ALK*, and therefore requires extra copies to exert an equivalent cellular effect. The phenomenon may be analogous to the consistent amplification of the *PAX7-FKHR* variant fusion gene in alveolar rhabdomyosarcoma.^{36,37}

ATIC-ALK may make up a significant proportion of variant *ALK* fusions. In the present series, *ATIC-ALK* accounted for 2 of 15 (13%) cases positive for *ALK* by immunostaining or for *NPM-ALK* by RT-PCR. Considering the cytogenetic reports of the inv(2)(p23q35), three cases of *ATIC-ALK* have been previously reported.²⁶ Aside from *TPM3-ALK*, reported in three cases,²⁸ no other known *ALK* variant translocations have been recurrent.

As the third reported translocation partner of *ALK*, the finding of *ATIC* highlights the promiscuous nature of many genes involved in oncogenic translocations.³⁸ *NPM* also rearranges with other genes, resulting in the *NPM-RARα* fusion in rare cases of acute promyelocytic leukemia and the *NPM-MLF1* fusion seen in some cases of myelodysplastic syndrome and acute myeloid leukemia.^{39,40} In ALCL, no variant fusions involving *NPM* but not *ALK* have so far been identified, although there are cytogenetic case reports of a t(3;5)(q12;q35)⁷ and a t(1;5)(q32;q35).⁴¹ In the absence of molecular studies, however, the latter two cases may simply contain a cryptic complex t(2;5). Interestingly, a recent report has also implicated *ALK* activation by rearrangement as a recurrent alteration in a mesenchymal tumor, the inflammatory myofibroblastic tumor.⁴²

The potential clinical significance of these variant *ALK* fusions is that *ALK*+ ALCL, defined either by immunohistochemistry, or by molecular or cytogenetic detection of *NPM-ALK*, is a prognostically favorable subset of ALCL. Before the development and application of *ALK* immunodetection, this important observation was statistically hampered by the limited numbers of cases with cytogenetic or molecular data.^{1,43,44} Recent retrospective analyses of large series of ALCL by *ALK* immunostaining have established that *ALK*+ ALCL occurs in significantly younger patients, is more often extranodal, and has a markedly better clinical outcome.^{22,45,46} Furthermore, a recent multivariate analysis indicates that the survival advantage of patients with *ALK*+ ALCL is not merely secondary to their younger age.⁴⁶ Although this accounts for

the bimodal age distribution of Ki-1 ALCL, the age ranges for ALK+ and ALK- cases still overlap considerably.

Pathologically, ALK+ ALCL are of non-B cell lineage and almost always coexpress CD30 and EMA.^{21,46} However, ALK+ ALCL may be morphologically indistinguishable from ALK- cases. *ALK* expression crosses all morphological forms of ALCL, including appearances which are neither anaplastic nor large cell.^{21,22,47} Hence, the simpler term ALK+ NHL has been proposed by some.^{21,22} In these clinicopathological studies of ALK+ ALCL, cases with variant *ALK* fusions have been lumped with the more common *NPM-ALK* cases. Although it is reasonable to expect that their clinical behavior may be closer to that of *NPM-ALK*+ ALCL than ALCL lacking any *ALK* alterations, it is only with the cloning of these variant *ALK* fusions that a systematic clinical comparison becomes possible.

Finally, it is tempting to speculate about possible therapeutic implications of the *AT1C-ALK* fusion. The AICARFT reaction mediated by AT1C is a folate-dependent reaction, and as such is thought to account in part for the anti-purine effects of antifolates such as methotrexate whose primary target is dihydrofolate reductase.³⁴ This raises the intriguing possibility that, as the *AT1C-ALK* fusion represents a reciprocal rearrangement (leaving only one normal *AT1C* allele/cell), Ki-1 ALCL bearing this genetic alteration could be more sensitive to antifolates because of lower cellular AT1C activity, due to haploinsufficiency and/or potential dominant negative effects of heterodimerization of AT1C-ALK with residual native AT1C.

Note added in proof: Since our study was completed, Ma et al⁴⁸ have reported in abstract form the independent cloning of the identical *AT1C-ALK* fusion and its detection in five cases with the inv(2)(p23q35).

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